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Detection of mutations in DNA

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Methods for detecting known and unknown mutations are becoming increasingly important as new disease genes are identified and new mutations are found in them. These methods are also expensive and time consuming. Over the past year major efforts have been directed towards developing new assays and making current assays faster and cheaper.

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Introduction

The detection of mutations is becoming increasingly important for several reasons. Firstly, more and more genes are being identified as the cause of specific diseases and the mutations of these genes need to be identified to prove the causal relationship (for example, cancer is increasingly being shown to be a result of mutations in oncogenes and tumour suppressor genes). Secondly, clinicians need to know which mutations cause the disease in particular patients for diagnostic purposes. Also, the pathogenicity and epidemiology of infectious agents can be correlated with specific sequence changes.

All these mutations could be (and many are) detected by DNA sequencing. Although sequencing has become easier in recent years due to the advent of polymerase chain reaction (PCR) technology, once a mutation in a particular gene is identified as a causative agent of a particular disease, specific methods are usually used to search for this mutation in patients with the disease. These methods will be referred to as diagnostic methods.

Methods have been developed to avoid the need to completely sequence a piece of DNA to detect unknown mutations. These methods, known as screening or scanning methods, localize the mutation so that only a short stretch of DNA has to be sequenced. Analysis of papers published in *Genomics* and the *American Journal of Human Genetics* in the past year indicates that 33% and 16% (respectively) of the papers describing the identification of previously unknown mutations, used screening methods.

Diagnostic methods are most useful in the detection of inherited diseases in which a few known mutations account for all cases (e.g. one mutation accounts for all known cases of sickle cell disease). They are less useful in diseases that result from many mutations. An extreme case of this is haemophilia B which results from hundreds of different mutations. Thus in this situation, new cases really need a screening method even though causative mutations are known. There are intermediate situations, such as the thalassaemias where most mutations that are causal in a particular race are known. In this case one has the choice of using a diagnostic method, such as allele-specific oligonucleotides (ASO) for known mutations and then sequencing if a mutation is not found, or using a screening method.

The methods to be considered in this review will be those used to detect primarily point mutations, small deletions and insertions. These have been recently reviewed [1–4]; and the origin of the methods and the technical details can be found in these references. This review will concentrate on new methods, as well as, novel or substantial applications or modifications of current methods. This will not include sequencing which is covered by Landegren (pp 12–17) in this issue. Also, probes, automation and PCR are covered by Landegren (pp 12–17) and Markham (pp 8–11). The screening methods that I will review here are RNase, chemical cleavage of mismatch (CCM), carbodiimide, denaturing gradient gel electrophoresis (DGGE), single-strand conformational polymorphism (SSCP) and the heteroduplex methods. The diagnostic methods include ASO, allele-specific amplification (ASA), primer extension, creation of a restriction enzyme site by mismatched PCR and ligation. All these methods are currently being actively used.

Screening methods

RNase A

RNase A analysis is one of two early screening methods [5] dependent on a simple RNase A cleavage at a mismatch site in an RNA probe hybridized to mutant target DNA (or RNA). Despite the fact that only 70% of mutations are detected [5] using this method, and that the RNA probes have to be manufactured, it is still being actively used. The ability to work with genomic DNA is a specific advantage of a method called 'exon scanning'

Abbreviations

ASA—allele-specific ampflification; **ASO**—allele-specific oligonucleotide; **A2T**—3 azido-3-deoxythymidine; **CCM**—chemical cleavage of mismatch; **DGGE**—denaturing gradient gel electrophoresis; **PCR**—polymerase chain reaction; **SSCP**—single-strand conformation polymorphism; **TGGE**—temperature gradient gel electrophoresis.

[6••]. Here a wild-type cRNA probe is hybridised with unamplified mutant genomic DNA so that RNase cleaves the RNA at mismatches (cleavage also occurs at exon junctions as genomic introns loop out). Although some results were false positives and some insertions were not detected using RNase cleavage, application of this method to globin gene scanning showed that a higher proportion of mutations were detected than had previously been predicted [5]. A major topical application was to studying the variation of human immunodeficiency virus isolates [7•]. Patterns were identified according to geographical distribution and temporal appearance of mutations associated with the acquisition of resistance to AZT. A further topical application was to the *apc* gene in colorectal cancer [8•].

Gradient gel electrophoresis

The other early screening method developed was DGGE [9]. Double stranded (homoduplex or ideally heteroduplex) DNA is electrophoresed into a denaturant of increasing concentration until the last domain is denatured, and the movement of the DNA down the gel is arrested. DNA sequences differing by a single base pair will stop at different points, thus the mutation can be detected in most cases. A recent extension of this method [10••] allows study of unamplified genomic DNA and features two improvements, the GC clamp and the heteroduplex. This involves restriction enzyme digestion of genomic DNA, followed by hybridization with a labelled single stranded probe containing a GC rich region to produce heteroduplexes, extension from the probe to include the region to be assayed using DNA polymerase and then electrophoretic analysis. The fact that secondary structure of the DNA under the electrophoretic conditions might give rise to unpredicted melting behaviour in some cases was suggested [11•]. This could then be used to detect mutations because the pattern of its gel is altered.

One of the advantages of DGGE was illustrated in the analysis of error rates of three DNA polymerases [12•]. This advantage is the ability of the method to detect mutant molecules which are present at very low levels (only a few percent) after separation. When mutant molecules were premixed at a concentration of 1%, amplification with two of the polymerases showed a detectable band with DGGE, but the third did not. In a paper using the related method, temperature gradient gel electrophoresis (TGGE) [13•], quantitation of template number was also achieved via the PCR. An internal standard differing from the template to be quantitated by a single base is added, before PCR. After PCR, and the subsequent addition of a small amount of labelled standard, the mixture is separated by TGGE, and then melted and reannealed. The labelled standard then forms homoduplexes or heteroduplexes depending on whether it binds to self or to its template sequences respectively. These two duplexes can be separated and quantitated. A novel variation is the gradual and uniform raising of the temperature of the plate during electrophoresis [14•]. This technique, referred to as temperature sweep gel electrophoresis, is advantageous because the gel can be poured simply without gradient and the equipment is a normal thermo jacketed vertical-slab gel apparatus.

The application of a screening method, such as DGGE, in the diagnosis of patients with β-thalassaemia was reported by Losekoot *et al.* [15••]. Statistics show that 20% of the recorded cases in Holland cannot be diagnosed using the conventional ASO approach. To avoid sequencing, DGGE was applied in all cases, to a region in which 90% of all mutations had been described. It is possible that these methods may be covered by a recent patent [P1•] which specifies homo- and heteroduplex formation of amplified polymorphic regions and then separation, and also another patent [P2•] which covers resolution of mutant and non-mutant DNA.

Chemical cleavage of mismatch

The CCM method relies on the differential reactivity of mismatched C and T bases to hydroxylamine and osmium tetroxide respectively, in heteroduplexes between mutant and wild-type DNA (or RNA). The position of the mismatch, and hence the mutation, is defined by sizing on gel electrophoresis after cleavage at the reactive position by piperidine.

The realization that a small number of TG mismatches were unreactive with osmium teroxide and that the respective causative mutations would be missed [16••], led to the finding that such mutations could be detected using a mutant probe that converts the TG mismatch to a CA mismatch which is readily detectable by hydroxylamine. This suggested that to detect all mutations the amount of work would have to be doubled. It was shown, however, in the *Caenorhabitis elegans* system [17•] that mutations could readily be detected when mutant and wild-type were added (both labelled) together in equivalent quantities rather than in the 10-fold excess of unlabelled mutant as originally recommended. This meant that probes of mutant and wild-type of both senses could be tested in the one tube with one chemical. This strategy means that each mutation has two chances of being detected as a reactive mismatched base on different strands and hence in different contexts. This makes it extremely unlikely that any mutation will be missed, putting it in the same category as sequencing used as a screening method.

Potassium permanganate has been suggested as an alternative chemical to osmium tetroxide [18•] for mismatched T bases but it has to be used in association with noxious tetra-alkylammonium salts. Potassium permanganate alone does not give the discrimination that osmium tetroxide does between matched and mismatched bases (R. Cotton unpublished results). Notable applications have been in the study of the *p53* gene in colorectal cancer [19•] and the globin gene in β thalassaemia [20•].

Single strand conformation polymorphism

One of the simplest methods for screening for mutations is SSCP. DNA is denatured and then immediately run on a non-denaturing gel. The secondary structures of wild-type single strands or mutant single strands differ-

ing by a single base are usually different which leads to a different rate of migration in the gel and hence detection of the mutation. To improve the method, which works best and misses least mutations with 200–400 base pair lengths, a strategy was described for longer lengths such as 2.7 kb [21•]. The fragment was cut with frequent cutter enzymes and run first in one dimension in a denaturing gel and then in a second dimension on a non-denaturing gel. Mutant and wild-type gels are then compared but only one sample can be run per gel.

The adaption of the Pharmacia 'Phast system' to SSCP with silver staining [22•,23•] with precast gels appears to promise better quality bands and thus better mutation detection, together with the elimination of the need to use radioactivity. This method may be covered by a patent [P2•] as it resolves mutant and non-mutant DNA.

As with the DGGE system the separation of mutant molecules from wild type allows them to be quantitated or studied separately. A mutant strand (as little as 3%) can be amplified for sequencing [24•] or sequenced directly [25•] using SSCP. A major application [26••] of the method was in the detection of mutations in the *p53* gene where it was shown not only to detect a mutation but also to simultaneously detect loss of one of the two alleles. A comparison of the SSCP and heteroduplex methods will be mentioned below.

Carbodiimide

The carbodiimide method relies on the reactivity of a water soluble carbodiimide with mismatched G or T bases. A recent modification [27••] has allowed a much more convenient analysis of this reactivity and hence detection of the position of mutations. Heteroduplexes are formed between equimolar portions of mutant and wild-type DNA reacted with carbodiimide. Primers are then used for extension from each end of the duplexes. Where the carbodiimide has reacted, extension is arrested. This shortened product is detected after electrophoresis. The method allows two chances for a mutation to be detected, and has been used to detect unknown mutants [28•].

Heteroduplex method

Like SSCP this method is one of extreme simplicity. Heteroduplexes containing a single-base mismatch can be accurately separated from the related homoduplexes, on non-denaturing 'hydrolink' gels [29••]. Thus, in a heterozygous individual, detection of a mutation simply involves PCR, an extra denaturing/reannealing step, running the product on the gel and staining with ethidium bromide. The size of the fragments screened were 211 and 420 base pairs. It is possible that this method is also covered by the patent [P1•] mentioned above. A major application of this method has been in the study of the rhodopsin gene in autosomal dominant retinitis pigmentosa [30•]. Four new mutations were detected in this study.

The SSCP and the heteroduplex method were compared [31••]. This study showed that the heteroduplex method detected eight of nine point mutations, but only two of these mutations were detected by SSCP. This is presumably because the DNA used formed a hairpin loop, the presence of which has little effect on secondary structure. The mutations were engineered to be in the loop. Because the principles of the two methods are different and they are both simple, it is suggested they be used in combination.

Other methods

The 'PCR fingerprinting' method [32••] offers a rapid method of matching *bla* alleles it works on a similar principle to the heteroduplex method above and could be placed in the same category. The method is a specific assay of genes that have pseudogenes. In the case of *bla-dr* genes for an individual homozygous at the locus, after PCR analysis, not only the gene but also the pseudogene is amplified. The pseudogene and gene products form heteroduplexes during PCR such that when analyzed on a 12% non-denaturing polyacrylamide gel, bands appear representing the specific homoduplexes and heteroduplexes, as the mismatches in the heteroduplexes retard them. An individual homozygous for another allele will have a different set of heteroduplexes and hence a different pattern. This means the pattern can be used to 'type' the allele. Heterozygotes at the locus will have more complex patterns. This simple method has allowed matching of the unrelated donors in 8 hours [33•] and will make a considerable difference to the efficiency of matching.

When inbred strains of mice have an inherited disorder it is a challenge to identify the causative defective gene. In order to study diseases in which there are specific alleles with high reversion rates, a method has been developed which has been shown to identify the defective locus [34••]. The method is based on the assumption that a reverting locus is repeated in tandem and genomic DNA yields a high copy number of repetitive DNA (1000 copies/genome) when it is probed using a southern blot analysis. This method, termed 'genome scanning', was able to identify an altered band which ultimately was identified as the 'pink eyed' mutation.

Diagnostic methods

Allele-specific oligonucleotides

This robust method has been used successfully for many years and is now widely applied. Two improvements have recently been suggested: firstly, a novel mode of attaching the oligonucleotide by a single point to a membrane with carbodiimide was described [35•]. This avoids the use of heat or UV light, which leads to a low hybridization efficiency; secondly, background hybridization to the normal allele has been largely eliminated by 'cold competition' (RB Wallace, personal communication) whereby unlabelled oligonucleotide complementary to the normal allele, if a mutant is being measured, is added to the hybridization mix. A patent has been published for the reverse blot in which different oligonucleotides are spotted to a membrane and the sample applied to this [P3•].

Allele-specific amplification

Originally described by several laboratories ASA is widely used due to its simplicity and rapidity. One of the pair of primers used for PCR is synthesized so that it has a mismatch with normal, but not with mutant sequences and the other a mismatch with mutant but not with normal. Thus under PCR conditions, particular primers will only amplify those complementary alleles which are present and thus assay simply for the presence of alleles.

The only novel modification of this method recently was a double ASA, simultaneously reported by two laboratories, [36•,37•] to decide whether two sequence variations are on the same chromosome or not. Essentially this means haplotyping can be achieved in the absence of DNA from relatives. This is done by adding allele-specific primers for both ends of the potential PCR product, and if they do not bind there is no product, which thus provides information as to whether or not the two variations are linked and their nature by using of all the possible allele specific primer combinations.

There have been many applications of ASA but the most notable has been in the detection of mutations in β thalassaemia [38•] where 17 different mutations were detected in 100 first trimester prenatal diagnoses.

Ligation assay

In this assay, two abutting oligonucleotides are synthesized in the area of a variation so that the 3′ base of the primer that is nearest to the 5′ end of the sequence, lies on the position of the variation. This 5′ oligonucleotide is then synthesized to be complementary to the two variants. When ligase is added and there is no mismatch, ligation occurs. There is no ligation where there is a mismatch and this event can be readily detected.

The ligation assay has now been converted to an automated non isotopic form [39••]. The assay is in a colorimetric enzyme-limited immunosorbent assay (ELISA) format and used in a robotic work station. The application has been used for the diagnosis of common genetic diseases. A further development has been the isolation of a thermostable ligase [40•]. This has been used in association with PCR so that the assay both amplifies the DNA and discriminates a single base substitution making the outcome similar to ASA.

Primer extension

Base variation can be detected by the application of a primer synthesized so that it abutts onto but does not cover the base to be assayed. The primer is extended in separate tubes, with only the two labelled bases, complementary to those being assayed at the position. Depending on which allele is present one or the other or both are incorporated. Further descriptions of the method include an electrophoretic assay [41•] or a colorimetric assay [42•] which makes the system amenable to automation. A patent covering this simple technology has appeared [P4•].

Artificial introduction of restriction sites by PCR

Mismatched primers are used to introduce artificial restriction sites into the region of a sequence variation. When the base is changed due to mutation restriction enzyme susceptability is changed [43]. This allows an extremely simple assay to be performed. One allele is cut by the enzyme and the other is not. In addition to increasing the number of applications of this method an interesting modificiation made by two laboratories allows the amplification of mutant alleles, thus enabling their detection, if they are present at a frequency of 1 in 1000 [44•,45]. In this method an artificial restriction site is introduced at the point of the mutation, at a position near to, but inside, a natural invariant sequence used in a first round of PCR. The DNA is then digested and in this case wild-type allele is digested and is unavailable for a second round of amplification using the same primer in the region of the mutation and a different primer inside the other initial primer. This then prevents amplification of the DNA of the artificially cut allele after the first PCR cycle, thus allowing differential enrichment of the mutant sequence. A patent relating to this technology has recently been published [P5•]. This covers the detection of a mutation in an allele using a specific restriction enzyme.

Conlusion

The worker interested in detecting mutations has an increasing number of improving technologies to choose from. When new mutations are sought the simplest methods of analysis are the heteroduplex, SSCP and DGGE methods, but an added acertainty of detection is given by CCM and Ccarbodiimide. The separative potential of the former group makes them especially useful for some applications. For known mutations the major thrust is the development of methods which can performed at an automated work station whereas ASO, ASA and the artificial introduction of restriction sites are very popular in research and clinical laboratories.

Acknowledgements

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40. • BARANY F: **Genetic Disease Detection and DNA Amplification Using Cloned Thermostable Ligase.** *Proc Natl Acad Sci U S A* 1991 88:189–193.

Important in allowing amplification and ligation to occur in the one tube.

41. • KUPPUSWAMY MN, HOFFMANN JW, KASPER CK, SPITZER SG, GROCE SL, BAJAJ SP: **Single Nucleotide Primer Extension to Detect Genetic Diseases: Experimental Application to Hemophilia B (Factor IX) and Cystic Fibrosis Genes.** *Proc Natl Acad Sci U S A* 1991 88:1143–1147.

Expanded description of an earlier report.

42. • SYVANEN AC, AALTO-SETALA K, HARJU L, KONTULA K, SODERLUND H: **A Primer-guided Nucleotide Incorporation Assay in the Genotyping of Apolipoprotein E.** *Genomics* 1990 8:684–692.

Development of the primer extension method which makes it amenable to automation.

43. COHEN JB, LEVINSON AD: **A Point Mutation in the Last Intron Responsible for Increased Expression and Transforming Activity of the c-Ha-*ras* Oncogene.** *Nature* 1988 334:119–121.

44. • CHEN J, VIOLA MV: **A Method to Detect *Ras* Point Mutation in Small Subpopulations of Cells.** *Anal Biochem* 1991, 195:51–56.

An important strategy to quantitate the amount of a minority allele that is particulary important in cancer. In this case a mutant RAS allele in 5% of cells could be detected.

45. • LEVI S, URBANO-ISPIZUA A, GILL R, THOMAS DM, GILBERTSON J, FOSTER C, MARSHALL CJ: **Multiple K-ras Codon 12 Mutations in Cholangiocarcinomas Demonstrated with a Sensitive Polymerase Chain Reaction Technique.** *Cancer Res* 1991, 51:3497–3502.

Another paper able to detect a mutated allele of *K-ras* in the presence of an excess of 1000 times that of the normal allele.

Annotated Patents

- • of interest
- •• of outstanding interest

P1. • LIFECODES CORP: **Genetic analysis of nucleic acid by forming homo- and heteroduplex of amplified polymorphic region, then separation, used to diagnose genetic disease.** 5/5/89 89US-348350. 15/11/90 WO9013668 A.

This could cover any method which relied on heteroduplex formation and subsequent separartion for example the DGGE and Heteroduplex methods.

P2. • MASSACHUSETTS INSTITUTE TECHNOLOGY: **Resolution of mutant and non-mutant DNA which can obtain mutational spectra of DNA sequences, useful as diagnostic tool in assessing exposure to mutagens.** 13/7/89 89US-379087. 24/1/91 WO9100925.

This patent could cover the SCCP and DGGE methods.

P3. CANON KK: **Detecting nucleic acid by hybridisation with probe preferably immobilised with sample DNA carrying detectable label e.g.for diagnosing genetic disease.** 23/6/89 89JP-159717. 16/1/91 EP-407789 A. 13/2/91 EP-412883 A.

This patent could cover the reverse dot–blot version of the ASO

P4. BERTIN AND CIE: **Detecting single base in nucleic acid sequence by hybridisation with primer adjacent to the specific base, then incorporation of detectable modified nucleotide.** 11/8/89 89FR-010802. 13/2/91 EP-412883 A.

This patent describes the principle of and probably covers the primer extension method.

P5. MASSACHUSETTS INSTITUTE TECHNOLOGY MCKENNA JJ: **Detecting mutation in allele, especially proto-oncogene using specific restriction enzyme or antibody.** 1/10/82 82US-432337. 27/12/90 EP-120958 B.

This patent may cover the artificial introduction of restriction site method.

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